Chiral Recognition of N-α-Acetyltryptophans with Molecularly Imprinted Polymeric Membranes Containing DVNE Derivatives

Masakazu YOSHIKAWA, * Takashi FUJISAWA, Jun-ichiro IZUMI, Toshio KITAO, and Shunji SAKAMOTO

Department of Polymer Science and Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606, Japan

Abstract: Molecularly imprinted polymeric membranes, bearing a tetrapeptide derivative H-Asp(OcHex)-Val-Asn-Glu(OBzl)-CH\(_2\)-, were prepared by the membrane preparation (casting) process in the presence of print molecule Ac-L-Trp. The molecularly imprinted polymeric membranes thus obtained showed adsorption selectivity toward the print molecule. The tetrapeptide derivative in the imprinted membranes preferentially recognized Ac-L-Trp from racemic Ac-Trp solutions. The affinity constant between Ac-L-Trp and the chiral recognition site was determined to be 9.7 \times 10^3 \text{ mol}^{-1}\text{dm}^3 from the adsorption isotherm of Ac-L-Trp in the molecularly imprinted polymeric membrane. Enantioselective electrodialysis was achieved with the present membrane, and the L-isomer was permeated in preference to D-isomer.

1. Introduction

The development of a novel technique to introduce molecular recognition sites into materials, which can be employed as stationary phases in chromatography, sensors, membranes, catalysts, and so forth, is interesting and important to the pharmaceutical, food production, agricultural, and chemical industries. Molecular imprinting, first proposed by Prof. Wulff (1-3), is one of a number of promising techniques that introduce a molecular recognition site into polymeric materials (4-9). The present authors adopted an oligopeptide, which gives enormous diversity of combination, as a candidate molecule to form a molecular recognition site. It is, however, difficult to apply conventional molecular imprinting techniques to the present study. This led us to develop an alternative molecular imprinting technique (10-14); that is, the "molecular memory" of the substrate to be recognized is induced in the membrane at the same time that the polymeric construction is prepared from the polymer solution. So far, it has been reported that the recognition site formed in the polymeric membrane, which consisted of a tetrapeptide derivative, recognized L-amino acids from their racemic amino acid mixtures. Our attention is centered on the search for novel oligopeptides, which show high chiral selectivity toward α-amino acids. Because of this, a novel material containing a tetrapeptide, H-Asp(OcHex)-Val-Asn-Glu(OBzl)-CH\(_2\)- (DVNE-Resin), having asparagine (Asn) as a constituent, was prepared. It is thought that asparagine, containing a terminal amide group in place of a carboxylic acid, is expected to give one more hydrogen bond than the corresponding aspartic acid. The potential of chiral recognition of the imprinted membrane from DVNE-Resin was investigated.
2. Experimental

2.1 Materials

Protected amino acids, Boc-L-Asp(OcHex), Boc-L-Val, Boc-L-Asn, and Boc-L-Glu(OBzl) were kindly provided by Kyowa Hakko Kogyo Co., Ltd. Chloromethylated polystyrene resin (Cl-Resin) (1% divinylbenzene), which had a Cl content of 0.78 meq/g, and dicyclohexylcarbodiimide (DCC) were purchased from Peptide Institute, Inc., Osaka, Japan and used without further purification. Dichloromethane (15), N,N-dimethylformamide (DMF) (15), 1-methyl-2-pyrrolidinone (NMP) (15), trifluoroacetic acid (TFA) (15), diisopropylethylamine (DIEA) (16), and 2-propanol (15) were purified by the usual methods. A copolymer of acrylonitrile and styrene (AS), containing a weight fraction of acrylonitrile equal to 0.33, was kindly supplied by Ube Cycon, Ltd. Ac-L-Trp, Ac-D-Trp, sodium azide, and ethanol were used without purification. Distilled water was employed.

2.2 Preparation of Membrane Materials

The membrane materials were prepared by Merrifield's technique of solid phase peptide synthesis (17,18).

DVNE-Resin was prepared as follows; Boc-L-Glu (OBzl)-OCH2C6H4-resin was prepared from 1.37 g (4.06 × 10⁻³ mol) of Boc-L-Glu (OBzl) and 1.00 g (7.80 × 10⁻⁴ unit mol of chloromethyl moiety) of chloromethylated polystyrene resin (1% divinylbenzene) (19).

<table>
<thead>
<tr>
<th>Table 1 Schedule for performed anhydride coupling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>9</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>11</td>
</tr>
</tbody>
</table>

*Percentages express vol./vol. ratios.

2.3 Preparation of Molecularly Imprinted Membranes

Each polymeric membrane studied in the present article was prepared from tetrahydrofuran solution, containing corresponding components. Copolymer of acrylonitrile and styrene (AS), in which weight fraction of acrylonitrile was 0.33, was adopted as a membrane matrix because DVNE- and Boc-DVNE-Resins do not form membranes by themselves. Ac-L-Trp was employed as the print molecule.

The typical membrane preparation process will be described using that for Ac-L-Trp imprinted membrane Boc-L-Glu(OBzl)-OCH₂C₆H₄-resin (1.25g, 7.8 × 10⁻⁴ unit mol of Boc-L-Glu moiety) was placed in a manual reactor similar to one reported (20) and carried through the schedules (21) shown in Table 1. The “pre-mix” reaction mixture used in all other couplings (Table 1) was prepared as follows: 4.68 mmol of Boc-amino acid, except Boc-L-Asn, in 10 cm³ of CH₂Cl₂ was cooled at 0°C and mixed with 483 mg (2.34 mmol) of DCC in CH₂Cl₂ (5 cm³). In the case of Boc-L-Asn, 20 cm³ of CH₂Cl₂/NMP (1:1, vol/vol) was employed instead of 10 cm³ of CH₂Cl₂. After the mixture was stirred for 30 min at 0°C, the precipitate was filtered at ambient temperature and washed with 5 cm³ of CH₂Cl₂. The combined filtrate and washings were then immediately added to the resin manually. The completeness of coupling was monitored by a Kaiser test (22). Acylation was judged complete in coupling of Boc-L-Asp (OcHex) and Boc-L-Val after two cycles. In the case of Boc-L-Asn, three cycles of the coupling reaction were carried out before completeness. Following the schedule mentioned above, the polystyrene resin bearing the tetrapeptide derivative, Boc-Asp(OcHex)-Val-Asn-Glu(OBzl)-CH₂-, (Boc-DVNE-Resin) was obtained. The polystyrene resin bearing H-Asp(OcHex)-Val-Asn-Glu(OBzl)-CH₂- (DVNE-Resin) was derived from Boc-DVNE-Resin by treatment with trifluoroacetic acid in dichloromethane (21).

From the hydrolysis of polystyrene resin thus obtained and the derivatization with (dimethylamino)-azobenzensulfonyl chloride (23), the concentration of tetrapeptide derivative thus introduced into chloromethylated polystyrene resin was estimated to be 1.43 × 10⁻⁴ mol/g of Boc-DVNE-Resin and 1.45 × 10⁻⁴ mol/g of DVNE-Resin.
from DVNE-Resin and AS, in which the mole ratio of print molecule to DVNE derivative is 3.0:1.6 mg of print molecule Ac-L-Trp, which amounts to three times the DVNE derivatives in the resin, was dissolved in 3 cm³ of THF with 15 mg of the DVNE-Resin. Then 285 mg of AS was dissolved in the THF solution. 2 cm³ of the THF solution thus obtained was poured into a flat laboratory dish (8.9cm-diameter) and the solvent allowed to evaporate at ambient temperature for 24h. The resultant membrane was then dried at 50°C for additional 2h. After drying, the print molecule was extracted from the membranes by a large volume of methanol until the print molecule could be hardly detected in the methanol by UV analysis. For this case, it was found that approximately 87% of print molecules could be extracted from the membrane. Other membranes were prepared in a similar manner as mentioned above. Thicknesses of the membranes thus obtained was between 104 - 117 μm.

2.4 Adsorption of Racemic Mixture to the Membranes

The molecularly imprinted polymeric membranes from DVNE-Resin were immersed in solutions containing racemic mixtures of compound, which were identical to those studied by enantioselective electrodialysis. These were 10 cm³ of 50 vol.% aqueous ethanol solutions of racemic Trp or racemic Ac-Trp, concentrations being 1.0 mmol dm⁻³, and the membrane was allowed to equilibrate at 40°C for 216 h. 0.02 wt.% of sodium azide was added as a fungicide. Aliquots of the original solution and after 216 h were used for quantitative estimation by HPLC (JASCO PU 980) equipped with a UV detector (JASCO UV 970) by using a CROWNPAK CR (+) column (250 × 4.0 (i.d.) mm) (Daicel Chemical Ind., Ltd.) and aqueous perchloric acid solution as the eluent.

The amount of amino acid in the supernatant subtracted from the amount initially in the solution gave the amount of amino acid adsorbed by the membrane.

Adsorption selectivity $S_{L/D}$ is defined as

$$S_{L/D} = \frac{[(AA)_L]/[(AA)_D]}{[AA]_L/[AA]_D}$$

where $(AA)_L$ and $(AA)_D$ are the amounts of amino acid adsorbed in the membrane, and $[AA]_L$ and $[AA]_D$ denote the concentrations in the solution after equilibrium was reached, respectively.

2.5 Adsorption Isotherms of Ac-D-Trp and Ac-L-Trp

The membrane was immersed in various concentrations of pure Ac-D-Trp or Ac-L-Trp solution and allowed to equilibrate at 40°C for 216 h. 0.02 wt.% of sodium azide was added as a fungicide. The quantitative analyses were done as described above.

2.6 Enantioselective Electrodialysis

The membrane, with an effective membrane area of 3.0 cm², was fixed tightly with Parafilm between two chambers of a permeation cell. The volume of each chamber was 40 cm³. A 50 vol.% aqueous ethanol solution of racemic amino acids was placed in the both chambers of the permeation cell. Each concentration of racemic amino acid was fixed to be 1.0 mmol dm⁻³. The electrodialysis was carried out with a prescribed applied potential between platinum black electrodes (10 mm square; distance between the electrodes, 65 mm) at 40°C with stirring. Aliquots were drawn from the permeate side at each sampling time. The amounts of D- and L-isomers that permeated through the membrane were estimated as described above.

The flux value, $J$ (mol cm⁻² h⁻¹), is defined as

$$J = \frac{Q}{A \cdot t}$$

where $Q$ (mol) is the amount of permeated amino acid, $A$ (cm²) is the effective membrane area, and $t$ (h) is time.

The separation factor $\alpha_{L/D}$ is defined as the flux ratio $J_L/J_D$ divided by the concentration ratio $[Ac-L-Trp]/[Ac-D-Trp].$

$$\alpha_{L/D} = \frac{(J_L/J_D)}{([Ac-L-Trp]/[Ac-D-Trp])}$$

3. Results and Discussion

3.1 Adsorption Selectivity toward Racemic Ac-Trp

In Table 2, amounts of Ac-Trp's adsorbed in the molecularly imprinted polymeric membranes and adsorption selectivities for the imprinted membranes are summarized. As for the adsorbed amounts, they are given not only in absolute amounts but also in relative ones, which were converted to those of DVNE (or Boc-DVNE) derivative basis for convenience of the following discussion. In Figs.1 and 2, the results summarized in Table 2 are shown visually. All plots gave straight lines: that is, adsorbed amounts increased with the increase in the molecular imprinting ratio (print molecule) / (DVNE). As for the membrane,
Table 2 Adsorption of racemic amino acids in molecularly imprinted polymeric membranes

<table>
<thead>
<tr>
<th>Print molecule</th>
<th>(Print molecule)/(DVNE)</th>
<th>AA</th>
<th>$10^3 \langle AA \rangle_{mol}$</th>
<th>$(AA)_{mol}$/(DVNE)</th>
<th>$S_{A(L/D)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-L-Trp</td>
<td>3.0</td>
<td>Ac-D-Trp</td>
<td>1.22 ± 0.13</td>
<td>1.75</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac-L-Trp</td>
<td>1.35 ± 0.10</td>
<td>1.93</td>
<td></td>
</tr>
<tr>
<td>Ac-L-Trp</td>
<td>1.0</td>
<td>Ac-D-Trp</td>
<td>0.34 ± 0.11</td>
<td>0.49</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac-L-Trp</td>
<td>0.44 ± 0.12</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>Ac-L-Trp</td>
<td>0.5</td>
<td>Ac-D-Trp</td>
<td>0.16 ± 0.15</td>
<td>0.22</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac-L-Trp</td>
<td>0.23 ± 0.14</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Ac-L-Trp</td>
<td>0.25</td>
<td>Ac-D-Trp</td>
<td>0.05 ± 0.04</td>
<td>0.07</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac-L-Trp</td>
<td>0.12 ± 0.07</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Ac-L-Trp</td>
<td>0.125</td>
<td>Ac-D-Trp</td>
<td>0.03 ± 0.03</td>
<td>0.04</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac-L-Trp</td>
<td>0.08 ± 0.05</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>Ac-L-Trp</td>
<td>0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Ac-D-Trp</td>
<td>0.20 ± 0.10</td>
<td>0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac-L-Trp</td>
<td>0.20 ± 0.10</td>
<td>0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Ac-D-Trp</td>
<td>3.0</td>
<td>Ac-D-Trp</td>
<td>1.05 ± 0.11</td>
<td>1.50</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac-L-Trp</td>
<td>1.04 ± 0.09</td>
<td>1.49</td>
<td></td>
</tr>
<tr>
<td>Ac-D-Trp</td>
<td>1.0</td>
<td>Ac-D-Trp</td>
<td>0.35 ± 0.11</td>
<td>0.50</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac-L-Trp</td>
<td>0.35 ± 0.09</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Ac-D-Trp</td>
<td>0.5</td>
<td>Ac-D-Trp</td>
<td>0.18 ± 0.11</td>
<td>0.26</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac-L-Trp</td>
<td>0.18 ± 0.09</td>
<td>0.26</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Molecularly imprinted polymeric membranes were prepared from Boc-DVNE Resin and AS. Others were prepared from DVNE Resin and AS.

<sup>b</sup>The value is relative one, which was converted to that of the Boc-DVNE derivative basis.

which was imprinted by Ac-L-Trp, L-isomer was always incorporated in the membrane in preference to D-isomer, even though different molecular imprinting conditions were used. The excess amounts of L-isomer incorporated in the membranes were determined to be 0.17 DVNE derivative from the two curves given in Fig.1.

Fig. 2 shows the adsorption selectivity of Ac-D-Trp imprinted polymeric membranes. As observed in the molecularly imprinted polymeric membranes containing the DIDE derivative as the chiral recognition site (12), the membrane imprinted by D-isomer scarcely showed adsorption selectivity toward racemic Ac-Trp mixtures. In the present study, the DVNE derivative consisted of L-amino acids. From this it can be drawn that the membrane, containing tetrapeptide derivatives of L-amino acids and imprinted with a L-amino acid derivative, recognized L-isomer, while the chiral recognition site was hardly formed in the membrane, containing tetrapeptide derivatives of L-amino acids and imprinted with a D-isomer. In the present study, Ac-D-Trp in the membrane preparation process played an important role.
role as a porogen to form pores, which functioned as permeation paths, in molecularly imprinted membrane bearing DIDE derivative as a recognition site (10,12). It can also be concluded that Ac-L-Trp in the membrane preparation process is effective not only for the formation of the permeation path but also for the formation of the molecular recognition site. In other words, Ac-L-Trp worked, in the present study, as a porogen to form the permeation path and as a print molecule to shape the molecular recognition site. This suggests that the membrane, having an oligopeptide derivative, which consisted of D-amino acids, and imprinted by D-isomer, might show D-amino acid specificity.

In addition to these results, the membrane, which was prepared from Boc-DVNE-Resin, in which the terminal amino residue in DVNE derivative was protected by Boc, scarcely showed the potential for chiral recognition, as is seen in Table 2. This suggests that the presence of terminal amino residue in the DVNE derivative is essential to achieve chiral recognition of racemic amino acid derivatives.

3.2 Adsorption Isotherms of Ac-L-Trp and Ac-D-Trp

From the results summarized in Table 2 and Figs. 1 and 2 in the present study and those reported previously (11-13), it can be said that the chiral recognition site toward L-isomer was formed by the presence of print molecule Ac-L-Trp in the membrane preparation process. The tentative schemes for formation of molecularly imprinted polymeric membranes bearing DVNE derivatives and recognition of racemic amino acid mixtures are given in Fig. 3. The adsorption isotherms of Ac-L-Trp and Ac-D-Trp in Ac-L-Trp imprinted membrane, which had a molecular imprinting condition (Ac-L-Trp) / (DVNE) of 3.0, were investigated in order to study the substrate specificity of the recognition site that was formed by the presence of print molecule during the membrane preparation process.

The adsorption isotherm of Ac-D-Trp in Fig. 4 is a straight line passing through the origin, implying that
Adsorption isotherms of Ac-D-Trp and Ac-L-Trp on DVNE membrane imprinted by Ac-L-Trp. (The mole ratio of print molecule, Ac-L-Trp, to DVNE derivative in the membrane preparation process was fixed to be 3.0; $k_D = 2.4 \times 10^2$; $n = 0.21$; $K_c = 9.7 \times 10^3$ mol$^{-1}$ dm$^3$; [DVNE] = 0.14 mol dm$^{-3}$.)

Ac-D-Trp was adsorbed in the membrane without specific interaction with the membrane. The isotherm of Ac-D-Trp can be represented by the following equation:

$$C_D = k_D [Ac-D-Trp]$$

where $C_D$ is the concentration of Ac-D-Trp adsorbed by the membrane, $k_D$ denotes Henry's law adsorption constant, and $[Ac-D-Trp]$ is the Ac-D-Trp concentration in the solution equilibrated with the membrane.

On the other hand, the adsorption isotherm of Ac-L-Trp has a complicated profile, implying that the adsorption consists of non-specific adsorption represented by Henry's law adsorption and adsorption on specific recognition sites toward L-isomer similar to the dual sorption of gases (24-26). The concentration of Ac-L-Trp adsorbed in the membrane $C_L$ can be represented by the following equation:

$$C_L = k_D [Ac-L-Trp] + nK_c [DVNE] [Ac-L-Trp] / (1 + K_c [Ac-L-Trp])$$

where $n$ is the ratio of the maximum amount of Ac-L-Trp adsorbed on the chiral recognition site to the amount of DVNE derivative in the membrane. $K_c$ is the affinity constant between Ac-L-Trp and the recognition site, [DVNE] is the concentration of DVNE derivatives in the membrane, and [Ac-L-Trp] denotes the Ac-L-Trp concentration in the solution equilibrated with the membrane. Here, it is assumed that DVNE derivative could be mostly found on the surface of the pore formed in the membrane as shown in Fig. 3. In the present study, the concentration of DVNE derivative in the membrane was determined to be 0.14 mol dm$^{-3}$. The three parameters for adsorption isotherms determined to fit best are as follows: $k_D = 2.4 \times 10^2$; $n = 0.21$; and $K_c = 9.7 \times 10^3$ mol$^{-1}$ dm$^3$. The value of 0.21 also supports the results of selective adsorption shown in Fig. 1, implying that 0.17 DVNE derivative in the membrane formed a chiral recognition sites that were able to recognize Ac-L-Trp from racemic Ac-Trp mixtures. From Fig. 3, one more important conclusion is that the chiral recognition site in the membrane exclusively recognized L-isomer and D-isomer was not incorporated in the recognition site.

In addition to these results, the membrane, which was prepared from Boc-DVNE-Resin, in which amino terminal residue in DVNE derivative was protected by protecting group of Boc, scarcely showed any
membrane chiral recognition, which is summarized in Table 2. From this it can be deduced that the chiral recognition was attained by the interaction between the carboxyl moiety on the substrate and the amino terminal residues resulting from the DVNE derivative, and the absolute configuration of side chain of Trp.

3.3 Enantioselective Electrodialysis

It is an interesting and important subject to develop applications for molecularly imprinted polymeric membranes in the chemical industry. The application of molecular imprinting to membrane separation is one of its most promising applications. The electrodialysis of racemic amino acids through molecularly imprinted polymeric membranes can be done using the permselectivity of the molecules which is reflected by their adsorption selectivities (11-14). Enantioselective electrodialysis of racemic Ac-Trp mixtures through use of the present membrane was studied in connection with the application of molecularly imprinted polymeric membranes to chemical industry. The effect of applied potential difference on enantioselective electrodialysis of Ac-Trp’s is shown in Fig. 5. The total flux was linearly proportional to the applied potential difference $\Delta E$. Over 10.0 V of $\Delta E$, enantioselective permeation was scarcely observed. However, the separation factor toward Ac-L-Trp increased with the decrease in $\Delta E$, and below 2.5 V of $\Delta E$, the separation factor reached 2.7, which was equal to its adsorption selectivity. The optimized potential difference can make it possible to attain enantioselective permeation of Ac-L-Trp, which should be reflected by its adsorption selectivity. However, the relatively high potential difference was too much for enantioselective permeation to be reflected in the adsorption selectivity and, as a result, the permselectivity of the membrane decreased with the increase in the applied potential difference. At last, permselectivity reached unity at over 10.0 V of $\Delta E$ in the present membrane system.

4. Conclusion

Molecularly imprinted polymeric membranes, bearing tetrapeptide derivative, was prepared during the membrane preparation (casting) process in the presence of a print molecule. The tetrapeptide derivative in the molecularly imprinted polymeric membranes formed chiral recognition sites, which recognized L-amino acid exclusively. The membrane, containing tetrapeptide residues of L-amino acids and imprinted with a L-amino acid derivative, recognized L-isomer in preference to D-isomer. However, the chiral recognition site was not formed by using D-isomer as the print molecule. Electrodialysis of racemic amino acids showed that the permselectivity of the membrane is directly reflected by its adsorption selectivity.

Acknowledgments

The present work was partly supported by the Ogasawara Foundation for the Promotion of Science & Engineering. The authors are grateful to Prof. Dr. Takeo Shimizu of Kansai Research Institute for his continuous encouragement. We would like to express our gratitude to Messrs. Kenichi Morooka and Kenji Soda for technical assistance.

References